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DOI: https://doi.org/10.15252/embj.201798873

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TCF/LEF dependent and independent transcriptional regulation of Wnt/β-catenin target genes

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Abstract

During canonical Wnt signalling, the activity of nuclear β-catenin is largely mediated by the TCF/LEF family of transcription factors. To challenge this view, we used the CRISPR/Cas9 genome editing approach to generate HEK 293T cell clones lacking all four TCF/LEF genes. By performing unbiased whole transcriptome sequencing analysis, we found that a subset of β-catenin transcriptional targets did not require TCF/LEF factors for their regulation. Consistent with this finding, we observed in a genome-wide analysis that β-catenin occupied specific genomic regions in the absence of TCF/LEF. Finally, we revealed the existence of a transcriptional activity of β-catenin that specifically appears when TCF/LEF factors are absent, and refer to this as β-catenin-GHOST response. Collectively, this study uncovers a previously neglected modus operandi of β-catenin that bypasses the TCF/LEF transcription factors.

Keywords signalling pathways; TCF/LEF; transcription factors; Wnt signalling; β-catenin

Subject Categories Signal Transduction; Transcription

DOI 10.15252/embj.201798873 | Received 19 December 2017 | Revised 19 September 2018 | Accepted 28 September 2018 | Published online 13 November 2018

The EMBO Journal (2019) 38: e98873

Introduction

Wnt signalling refers to a set of evolutionarily conserved signalling pathways which play important roles in initiating and regulating a diverse range of cellular activities, including cell proliferation, cell polarity, movement, differentiation, survival, self-renewal and calcium homeostasis (Logan & Nusse, 2004; Clevers, 2006; Städeli *et al*, 2006; Raymond Habas, 2008; Chien *et al*, 2009; Grumolato *et al*, 2010; Mohammed *et al*, 2016). The importance of so-called canonical Wnt signalling goes beyond embryonic development, as its constitutive activation has been implicated in the pathogenesis of several different cancers and hereditary diseases (Laudes, 2011; Clevers & Nusse, 2012; Polakis, 2012; Kahn, 2014).

The current model of canonical, β-catenin-mediated Wnt signalling is that in the absence of Wnt ligands, free cytoplasmic β-catenin is rapidly sequestered by a cytoplasmic "destruction complex" that consists of Axin, the adenomatous polyposis coli (APC) tumour suppressor protein, glycogen synthase kinase 3 (GSK3α and GSK3β, both referred to as GSK3) and casein kinase 1 (CK1). β-Catenin is subsequently marked for degradation by GSK3-dependent phosphorylation at key amino-terminal Ser and Thr residues (Mosimann *et al*, 2009; King *et al*, 2012). Wnt ligands inhibit the β-catenin "destruction complex", resulting in the accumulation of free cytoplasmic β-catenin, and its nuclear import (MacDonald *et al*, 2009; MacDonald & He, 2012). Within the nucleus, β-catenin specifically binds to proteins of the TCF/LEF (T-cell factor/lymphoid enhancer factor) family of transcription factors, in order to activate the transcription of Wnt target genes (van Amerongen, 2012; Niehrs, 2012).

Vertebrate genomes encode four TCF protein family members: three of them act preferentially as activators, TCF7, LEF1, and TCF7L2, and one as repressor, TCF7L1 (Hoppler & Kavanagh, 2007). The activation of target genes by the β-catenin/TCF complex has been established as the main *modus operandi* of canonical Wnt signalling (Cadigan & Waterman, 2012; Nusse & Clevers, 2017), and recent studies both in mammals (Schuijers *et al*, 2014) and in *Drosophila* (Franz *et al*, 2017) provide additional evidence that Wnt-dependent transcriptional output is predominantly executed by the partnership between TCF and β-catenin.

However, several studies have also suggested the existence of alternative interactors of β-catenin in various cell types. These include members of the SOX family (Kormish *et al*, 2009), FOXO proteins (Essers, 2005), the homeodomain protein PITX2 (Vadlamudi, 2005), hypoxia-inducible factor Iα (HIFIα) (Kaidi *et al*, 2007) and the bHLH protein MyoD (Kim *et al*, 2008). These findings, while awaiting independent confirmation, suggest the existence of one or more alternative branches within the canonical Wnt/β-catenin signalling pathway that are capable of bypassing TCF/LEF transcription factors. A difficulty in identifying TCF-independent functions of β-catenin could possibly

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arise due to the genetic redundancy of the four TCF/LEF encoding genes (Cadigan & Waterman, 2012), an aspect that rendered a genetic approach to this question practically inaccessible. Therefore, the discovery of TCF/LEF-independent branches might so far have been elusive due to the inability to simultaneously mutate or remove all TCF/LEF paralogous genes in a single cellular system, a technical challenge that, however, the advent of the CRISPR/Cas9 technique in human cells allowed us to overcome (Hsu *et al*, 2014; Sander & Joung, 2014; Moreira *et al*, 2017).

Here, we genetically engineered human HEK 293T cell clones that lack all four genes encoding TCF/LEF proteins or, alternatively, β-catenin. We used these tools to specifically test the existence of a β-catenin-mediated transcriptional activity that is independent of TCF/LEF factors. We present evidence for the existence of this phenomenon on two levels: (i) transcriptomics studies unveil genes whose expression rely on the presence of β-catenin but not of TCF/LEF factors, and (ii) despite its genome-wide DNA-binding profile being largely perturbed, β-catenin still exhibits physical association with chromosomes in the absence of TCF/LEF proteins. Intriguingly, our data reveal that, when TCF/LEF proteins are lacking, β-catenin embarks on the regulation of a different set of genes and binds alternative genomic locations. We refer to this phenomenon as the β-catenin-GHOST response. The transcriptional regulation of β-catenin-GHOST targets only occurs when TCF/LEF factors are absent, or when the β-catenin/TCF interaction is inhibited. We speculate that the β-catenin-GHOST response is mediated by crosstalk with other transcription factors, such as FOXO proteins.

Results

CRISPR/Cas9-mediated generation of TCF/LEF and β-catenin null cells

The TCF/LEF transcription factors are considered to act as main intermediaries of so-called canonical, β-catenin-mediated Wnt signalling (Clevers & Nusse, 2012). Recent high-throughput studies confirmed this tenet in *Drosophila* (Franz *et al*, 2017) and in mammalian cells (Schuijers *et al*, 2014). However, a few studies reported the ability of β-catenin to control gene transcription via interaction with non-TCF transcription factors. For example, β-catenin interacts with FOXO transcription factors, YAP1 and TBX5 in human cancer cells (Essers, 2005; Hoogeboom *et al*, 2008; Rosenbluh *et al*, 2012), and forms a complex with Oct4 to promote Oct4-driven pluripotency in embryonic stem cells (Kelly *et al*, 2011). Moreover, β-catenin cooperates in *Xenopus* with Sox17 in the activation of Sox17 target genes (Sinner, 2004). In addition, recent chromatin immunoprecipitation (ChIP) studies uncovered the possibility of genomic regions occupied by β-catenin but not by TCF7, TCF7L1, TCF7L2 or LEF1, indicating that recruitment of β-catenin via these transcription factors cannot account for the full extent of its genomic occupancy (Schuijers *et al*, 2014).

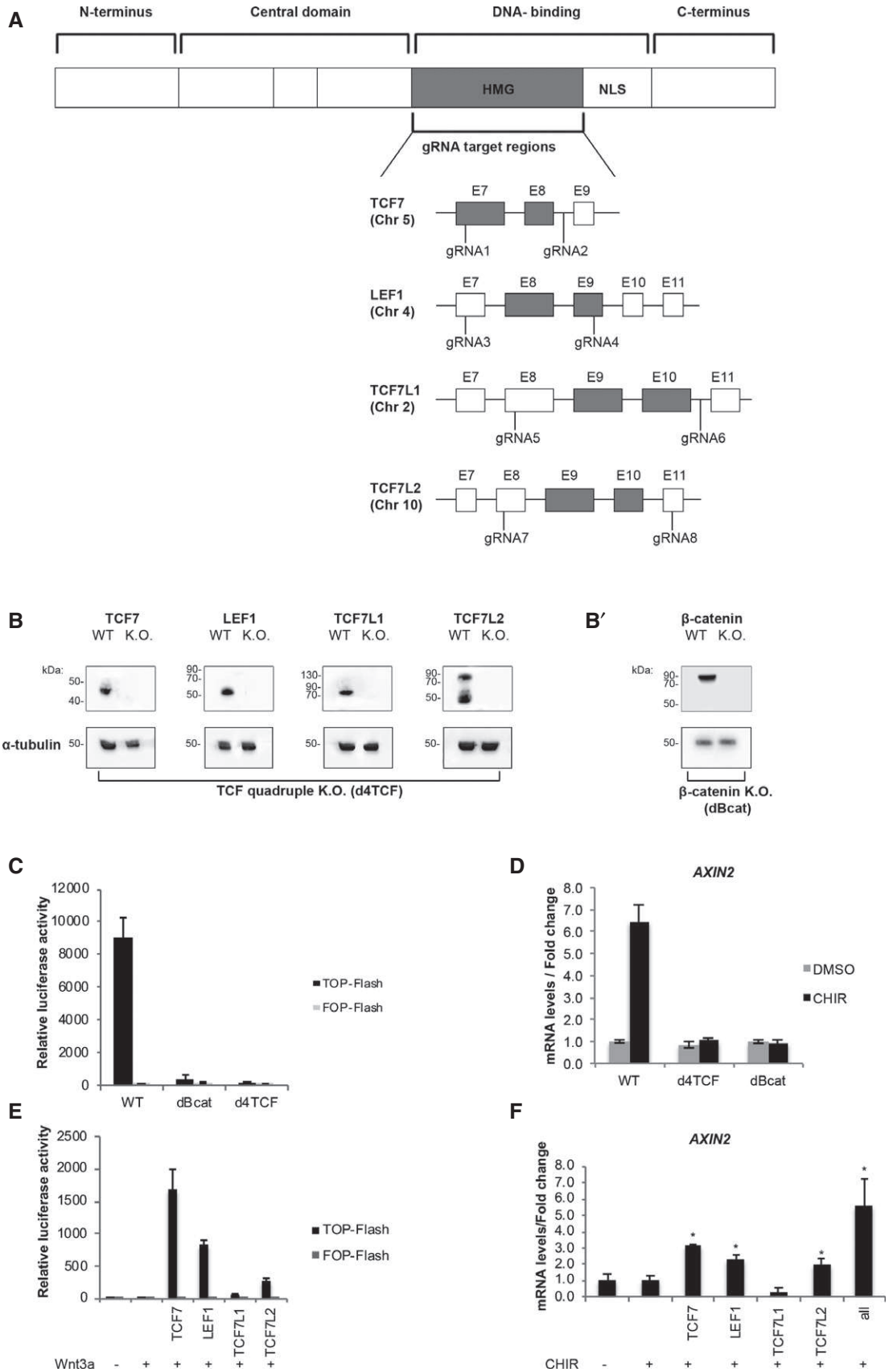
We reasoned that generating loss-of-function mutations of all four TCF/LEF genes in the same cellular system would constitute a powerful tool to conclusively test this. Taking advantage of CRISPR/Cas9 technology, we aimed at generating cell lines lacking all four TCF/LEF genes (Fig 1A). We chose human HEK 293T cells, an excellent model platform to study gene regulation downstream of β-catenin and TCF/LEF, for they are (i) widely used to study the biochemical events underlying the Wnt signalling pathway, (ii) do not carry pathway-specific activating mutations (Gujral & MacBeath, 2010) and (iii) are highly responsive to Wnt pathway activation, in that β-catenin only translocates into the nucleus when GSK3 is inhibited (Li *et al*, 2012). We confirmed that all TCF/LEF genes are expressed in our HEK 293T parental cells (Fig 1B). TCF/LEF transcription factors possess an evolutionarily conserved domain, the DNA-binding High-Mobility-Group (HMG) box: we designed two guide RNAs (gRNAs) spanning this region within each gene, in order to create large deletions (Fig 1A). This approach gave us confidence that such mutations would induce mRNA degradation via non-sense RNA decay (Brogna & Wen, 2009), or that truncated versions of the protein would not be able to interact with DNA, resulting in a loss of function. Using this strategy, we serially mutated the four TCF/LEF genes and generated a clonal quadruple knockout HEK 293T cell line (d4TCF) where TCF7, LEF1, TCFL1 and TCFL2 proteins were undetectable via Western blot analysis (Figs 1B and EV1). By exploiting the same CRISPR/Cas9-mediated strategy, we generated a clone of HEK 293T cells lacking β-catenin (dBcat). We designed two gRNAs targeting exon 4 and 12 of *CTNNB1*, the gene encoding for β-catenin, thereby creating a 11.5-kb deletion. The absence of β-catenin protein was confirmed by Western blot (Fig 1B′). In addition, the presence of the desired genomic deletions at these loci was confirmed by PCR, using primers flanking the regions near the gRNAs (Fig EV1).

d4TCF or dBcat cells display impaired canonical Wnt signalling

We set out to measure the responsiveness of d4TCF and dBcat cell lines to Wnt pathway stimulation. We transfected the widely used Wnt reporter TOP-Flash in parental control HEK 293T, dBcat and d4TCF cells and measured its transcriptional activity after Wnt pathway stimulation, achieved via the potent GSK3 inhibitor CHIRON99021 (hereafter referred to as CHIR) (Naujok *et al*, 2014). GSK3 activity leads to β-catenin degradation; CHIR is therefore a powerful activator of the Wnt pathway (Metcalfe & Bienz, 2011; Naujok *et al*, 2014). As expected, no luciferase activity was observed in both dBcat and the d4TCF cells, while control parental cells showed a strong, 8,000-fold reporter activation (Fig 1C). Consistently, quantitative RT–PCR (RT–qPCR) revealed that *AXIN2*, a prototypical Wnt target gene, was upregulated only in control but not in dBcat and d4TCF cells (Fig 1D). Other known canonical Wnt targets behaved in a similar manner (Fig EV2A). In a rescue experiment, transfection of individual TCF-expressing plasmids restores the ability of d4TCF cells to respond to Wnt3a (Figs 1E and EV2B) or CHIR stimulation (Figs 1F and EV2C). Interestingly, TCF7L1 failed to reactivate target genes, consistent with previous reports revealing an antagonistic relationship between canonical Wnt signalling and TCF7L1 on gene expression (Yi *et al*, 2011). These data showed that, in the absence of β-catenin or TCF/LEF factors, there is no detectable canonical Wnt signalling transcriptional response, as measured by TOP-Flash reporter and target gene activation.

d4TCF and dBcat cells are responsive to GSK3 inhibition

We set out to measure the transcriptional effects induced upon GSK3 inhibition (i.e. CHIR treatment) at a genome-wide level. We stimulated parental, dBcat and d4TCF cells for 24 h—the time


A
N-terminus
Central domain
DNA- binding
C-terminus
HMG
NLS
gRNA target regions
TCF7 (Chr 5)
E7 E8 E9
gRNA1 gRNA2
LEF1 (Chr 4)
E7 E8 E9 E10 E11
gRNA3 gRNA4
TCF7L1 (Chr 2)
E7 E8 E9 E10 E11
gRNA5 gRNA6
TCF7L2 (Chr 10)
E7 E8 E9 E10 E11
gRNA7 gRNA8
B
TCF7 WT K.O.
LEF1 WT K.O.
TCF7L1 WT K.O.
TCF7L2 WT K.O.
kDa:
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TCF quadruple K.O. (d4TCF)
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β-catenin WT K.O.
β-catenin K.O. (dBcat)
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Relative luciferase activity
TOP-Flash
FOP-Flash
WT dBcat d4TCF
D
AXIN2
mRNA levels / Fold change
DMSO
CHIR
WT d4TCF dBcat
E
Relative luciferase activity
TOP-Flash
FOP-Flash
Wnt3a
TCF7 LEF1 TCF7L1 TCF7L2
F
AXIN2
mRNA levels/Fold change
CHIR
TCF7 LEF1 TCF7L1 TCF7L2 all

Figure 1.

Figure 1. Quadruple TCF/LEF knockout (K.O.) HEK 293T cells are viable and proliferative but do not respond to Wnt pathway activation.

A Schematic representation of the TCF/LEF protein structure. Below, the guide RNAs (gRNAs) used for each specific gene are annotated. Grey boxes indicate the DNA-binding HMG box.

B Western blots for detection of TCF7, LEF1, TCF7L1, TCF7L2 and β-catenin (B′) proteins in wild-type (WT) and knockout (K.O.) cells. Uncropped version of the same Western blots is shown in Fig EV1.

C TCF-reporter TOP-flash assay performed on the indicated cell lines treated with 10 μM CHIR, for 24 h. The control FOP-flash reporter is not activated upon CHIR administration. The data represent the mean ± SEM of averages of nine independent experiments ($N = 9$). dBcat, β-catenin knockout cells; d4TCF, TCF/LEF quadruple knockout cells.

D Quantitative RT–PCR analyses of *AXIN2* transcripts, performed on the different cell lines treated with 10 μM CHIR or DMSO (control) for 24 h. Error bars show the standard deviation obtained from three independent experiments.

E, F Transfection of individual TCF-expressing plasmids could restore the ability of d4TCF cells to respond to Wnt3a (E), as measured in a TOP-flash assay, or of CHIR (F), as measured by *AXIN2* mRNA abundance. The data represent the mean ± SEM obtained from three independent experiments ($N = 3$). Samples were compared using Student's *t*-test. Asterisks (*) indicate a $P < 0.05$.

frame that showed the strongest activation of *AXIN2* (Fig EV2D)—and performed RNA extraction followed by deep sequencing (RNA-seq). We compared the transcriptomes of the following six conditions: unstimulated and CHIR-stimulated parental, dBcat and d4TCF cells (Fig 2A, Table EV1). The cell lines with different genotypes formed distinct clusters with and without CHIR treatment, indicating that dBcat and d4TCF cells behave reproducibly, but differently between them and vis-à-vis the parental cell line (Fig 2B). Notably, when compared to unstimulated wild-type parental cells (WT), d4TCF cells displayed significantly broader gene expression changes (ca. 1,400 deregulated genes) than dBcat cells (ca. 200 genes; compare Fig 2C and C′). This is consistent with the mechanism of action of TCF/LEF factors, which bind Wnt-responsive elements (WREs) even in the absence of active Wnt signalling (Nusse & Clevers, 2017); this, we believe, might have led to the large impact on gene expression in "Wnt-OFF" conditions. Upon GSK3 inhibition, we could detect broad transcriptional changes (upregulated and downregulated genes) both in dBcat and d4TCF cells (Fig 2D). We validated the expression changes displaying the highest fold change for each group via RT–qPCR (Fig 2E). However, as expected, CHIR failed to regulate canonical Wnt targets in dBcat and d4TCF cells (Figs 1D and 2A, and EV2A). Importantly, we generated three independent clonal cell lines for each genotype to exclude the possibility of a bottleneck effect (i.e. the generation of clonal cell populations, via single cells, might affect the overall behaviour of each clone) (Fig EV3A) and confirmed selected gene expression changes via RT–qPCR (Fig EV3B). Taken together, our data indicate that GSK3 inhibition leads to a transcriptional response both in the absence of β-catenin or TCF/LEF proteins.

Uncovering β-catenin-dependent transcriptional changes

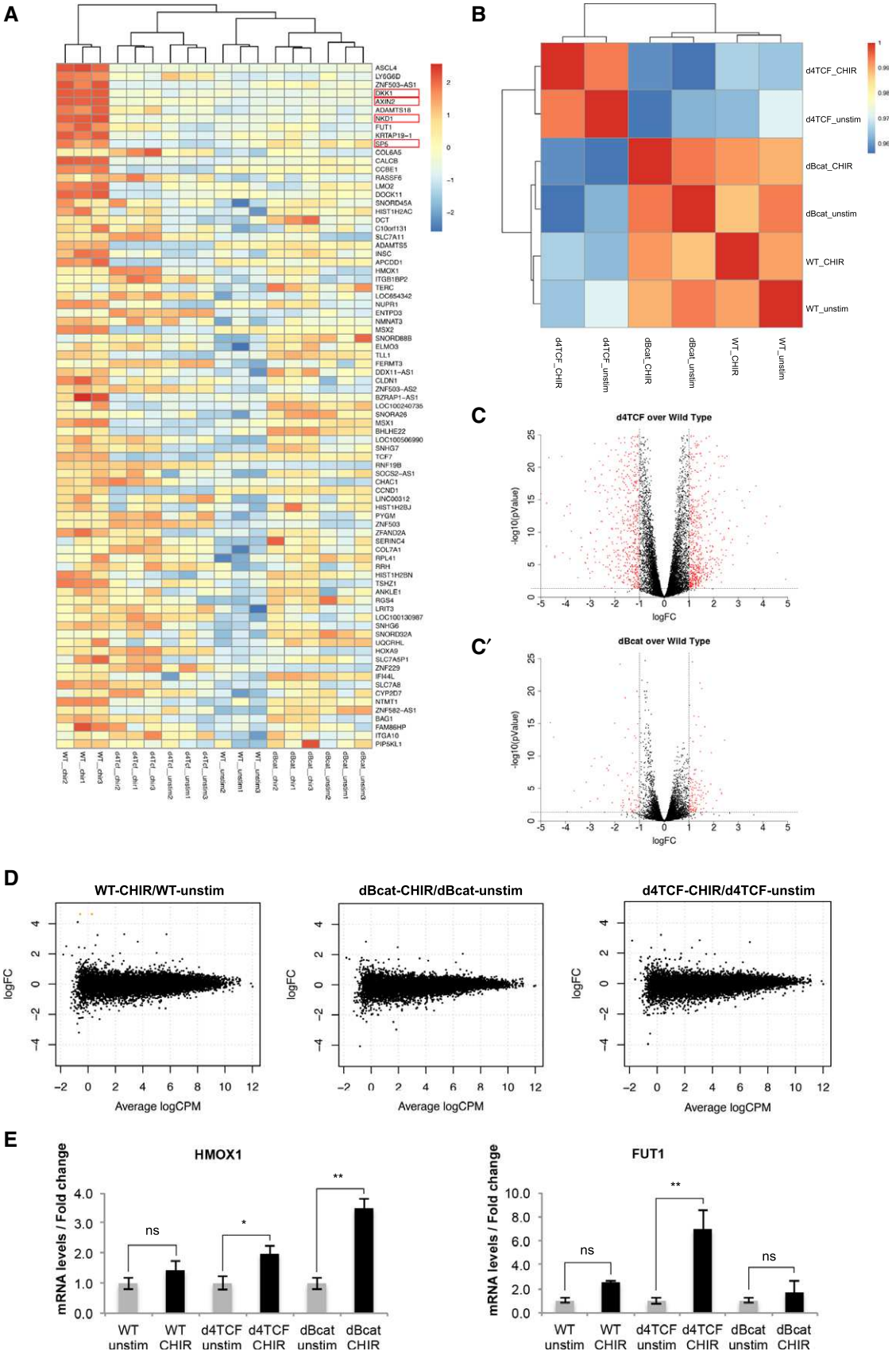
Under these experimental conditions, GSK3 inhibition in parental control cells reproducibly led to the altered expression of 231 genes (based on adjusted $P < 0.05$ and absolute log-fold change > 1), 81 of which were upregulated and 150 downregulated in three independent experiments (Figs 2A and 3A). GSK3 inhibition, however, has several effects by stabilizing a considerable proportion of the proteome—a phenomenon referred to as Wnt/STOP (Acebron *et al*, 2014; Koch *et al*, 2015). In order to distinguish those changes that strictly depend on β-catenin's activity from the other effects of GSK3 inhibition (e.g. the Wnt/STOP), we aimed at establishing a transcriptional signature of β-catenin. We reasoned that, among the changes induced by CHIR administration, those that depend on β-catenin should not vary when β-catenin is absent in dBcat cells. Of these 231 differentially expressed genes upon CHIR treatment, 166 were not regulated in dBcat cells (Fig 3A, red box), indicating that these transcriptional changes are a consequence of the nuclear transcriptional activity of β-catenin. We refer to this group of β-catenin-dependent changes as high-confidence β-catenin signature. Of note, this group includes, among the top ten upregulated genes, known canonical Wnt signalling targets such as *DKK1*, *AXIN2*, *NKD1* and *SP5* (Fig 2A red boxes, Fig 3A). We interpret this as a powerful validation of our approach.

d4TCF cells reveal TCF/LEF-independent β-catenin transcriptional activity

We focused on the high-confidence β-catenin-dependent transcriptional changes and asked whether some of these also occurred in d4TCF cells (Fig 3A). We identified a set of 27 β-catenin-dependent genes that are regulated by CHIR in d4TCF (based on adjusted $P < 0.05$ and absolute log-fold-change > 1). Therefore, we consider that ca. 15% of the 166 genes whose expression appears to depend on the presence of β-catenin do not require the activity of TCF/LEF transcription factors. With our cut-off values for gene expression, we could identify upregulated (four genes) and downregulated (23 genes) β-catenin-dependent but TCF/LEF-independent changes. These transcriptional changes were validated via RT–qPCR in several independent experiments (Figs 3B and C, and EV3C). In conclusion, in cells completely devoid of TCF/LEF proteins, β-catenin is still competent in regulating a subset of its target genes. This established that β-catenin can, in principle, bypass the activity of the TCF/LEF transcription factors in HEK 293T human cells.

β-Catenin physically occupies specific genomic regions in the absence of TCF/LEF transcription factors

If a subset of β-catenin target genes does not require TCF/LEF factors for their transcriptional regulation, it is plausible that β-catenin can interact with specific genomic regions independently of these factors. To test this, we adapted a chromatin immunoprecipitation (ChIP) protocol that enabled us to purify DNA-bound protein complexes (see Materials and Methods). We then performed ChIP using an anti-β-catenin antibody, followed by deep sequencing (ChIP-seq), on both CHIR-stimulated WT and d4TCF cells (Figs 4 and EV4). In WT, parental HEK 293T cells we identified ca. 1,300 high-confidence peaks, that were reproducible in 3 different experiments, even when different β-catenin antibodies were used, but


A
B
d4TCF_CHIR
d4TCF_unstim
dBcat_CHIR
dBcat_unstim
WT_CHIR
WT_unstim
C
d4TCF over Wild Type
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C′
dBcat over Wild Type
D
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dBcat-CHIR/dBcat-unstim
d4TCF-CHIR/d4TCF-unstim
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WT unstim
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d4TCF unstim
d4TCF CHIR
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dBcat CHIR

Figure 2.

Figure 2. RNA-seq analyses of CHIR-treated HEK 293T cells in the absence of TCF/LEF or β-catenin.

A Heat map showing the upregulated genes in wild-type (WT) parental HEK 293T cells upon CHIR treatment, ranked based on the log2 fold change. Increased and decreased transcription is depicted in red and blue, respectively. Gene names are indicated along the *y*-axis. Canonical Wnt targets are among the most upregulated genes in WT cells (red boxes). The genotypes of the cell clones used are indicated on the bottom of the heat map. Triplicates of each cell line are included.

B Hierarchical clustering of differentially expressed genes with at least a twofold change in any comparison. Dendrogram clustering indicates the relationships between the different experimental conditions.

C Volcano plot displays differentially regulated genes in d4TCF (C) and dBcat (C′) compared to WT parental cells. Red dots indicate significantly regulated genes based on adjusted *P*-value and log-fold change (logFC).

D Smear plots for every cell line indicating the behaviour of every gene after CHIR treatment. Differential gene expression is indicated by the log2 fold change (logFC), and the average logCPM (logarithm of counts per million reads) was used to estimate the accuracy of the expression level.

E RT–qPCR-based validation of three differentially expressed genes, upon CHIR stimulation in WT, dBcat or d4TCF cell lines. The data represent the mean ± SEM of averages of three independent experiments ($N = 3$). Samples were compared using Student's *t*-test. Asterisks (*) indicate a $P < 0.05$, and (**) a $P < 0.01$.

absent when ChIP was performed in dBcat cells (Figs 4 and EV4A, Table EV3). Several of these β-catenin-bound regions were previously characterized as WREs within the regulatory regions of canonical Wnt target genes, such as *AXIN2* and *LEF1* (Fig 4A). β-Catenin did not display any enrichment at known WREs in d4TCF cells (Fig 4A), confirming the requirement of TCF/LEF proteins for the correct positioning of β-catenin at these loci. Interestingly, some loci retained β-catenin enrichment in d4TCF cells (Fig 4B) even though genome-wide occupancy at TCF-bound regions was lost (Fig 4C) indicating that in the absence of TCF/LEF, β-catenin may associate with other transcription factors. While the number of TCF-independent β-catenin peaks appeared to be variable in different experiments, likely due to different pull-down efficiencies or perturbation of culture conditions (Fig EV4B, Table EV3), we identified a small subset of ca. 30 highly reproducible TCF-independent binding regions (Fig EV4C). In our analyses, we could not determine a direct relationship between TCF/LEF-independent peak-associated (ChIP-seq) and CHIR-regulated genes (RNA-seq). However, transcription factors binding does not necessarily occur at the cognate promoter of the genes, but could take place in distant enhancer regions (Dickel *et al*, 2013). We therefore defined a gene as "associated" when a ChIP peak is found within 50 kb from its transcriptional start site (TSS). This is the average distance of genes from CTCF peaks (~48 kb) and interacting promoters (Kim *et al*, 2007). The intersection between RNA-seq and ChIP-seq data showed that upregulated genes in WT were significantly more likely to be associated with β-catenin peaks in WT than expected by chance (3.987-fold increase) while genes upregulated in d4TCF cells were not (Fig EV4D). On the contrary, the fraction of downregulated genes in d4TCF was more likely to be associated with β-catenin binding upon CHIR stimulation (9.136-fold in d4TCF, 9.489-fold in WT, Fig EV4E). Importantly, while in WT cells β-catenin-dependent (2.083-fold) and TCF-dependent (2.388-fold) genes were more likely to have peaks in their proximity, in d4TCF cells, β-catenin-dependent but TCF-independent genes became the group with highest association with ChIP-seq peaks (52.236-fold increase, Fig EV4F). This suggests a strict requirement for TCF/LEF and β-catenin in WT cells, whereas β-catenin is relocated to TCF-independent target genes in d4TCF cells. Together, these findings indicate that in the absence of TCF/LEF, CHIR-stabilized β-catenin could embark on an alternate *modus operandi*, likely in association with other transcription factors. Indeed, whereas β-catenin-bound regions in wild type were highly enriched for the TCF consensus binding motif (Fig 4D), d4TCF cells only showed significant motif enrichment ($P < 1 \times 10^{-10}$, hypergeometric test) for Forkhead box (FOX) family of transcription factors (Fig 4E). Furthermore, all β-catenin peaks in d4TCF cells containing the TCF binding sequence also contained a FOX motif (Fig 4F), suggesting that β-catenin is recruited by FOX proteins in the absence of TCF/LEF.

β-Catenin activity in the absence of TCF/LEF: the β-catenin-GHOST response

Our RNA-seq and ChIP-seq results raised the possibility of a new β-catenin-dependent transcriptional program in the absence of TCF/LEF. We tested the existence of this by analysing the CHIR-dependent transcriptional response of d4TCF cells. CHIR-treated d4TCF cells displayed statistical changes in the transcription of 196 genes ($N = 3$, $P < 0.05$) (Fig 5A, Table EV2), and an unexpectedly large fraction of these changes (134, Fig 5A) occurred in d4TCF but not in control parental HEK 293T cells. Note that all changes were, by definition, independent of TCF/LEF proteins. Two-third of these gene expression changes (134) appeared to depend on β-catenin (Fig 5A), in that they did not occur in dBcat cells upon CHIR stimulation. Importantly, this list included the 27 β-catenin-dependent TCF/LEF-independent targets we previously identified (Fig 5A). The other 107 differentially expressed genes (Fig 5A, blue box) can therefore be considered as β-catenin targets, but their regulation occur only in the absence of TCF/LEF. It remained, however, possible that this group of 107 genes were not regulated by CHIR in dBcat because this mutant cell clone still possesses the TCF/LEF factors. To exclude this possibility and obtain conclusive evidence that β-catenin is required for their regulation specifically in a TCF/LEF quadruple knockout context, we introduced a loss-of-function mutation in *CTNNB1* in d4TCF cells, thereby generating a quintuple knockout cell line devoid of TCF/LEF and β-catenin proteins (we refer to this cell line as pentaKO). We performed RNA-seq of pentaKO cells ($N = 3$) and confirmed that at least 90 of the 107 β-catenin-dependent genes were either not upregulated in pentaKO ($n = 74$) or their transcript was undetectable ($n = 16$) indicating absence of transcriptional regulation. We refer to this set of 90 genes as the β-catenin-GHOST response (genes hidden outside the standard targets). We validated a subset of β-catenin-GHOST targets via RT–qPCR, showing that they respond to CHIR treatment in d4TCF cells but not in pentaKO cells, thereby conclusively confirming their dependence on β-catenin (Fig 5B, Table EV2).

The β-catenin-GHOST response was only detected upon the non-physiological removal of all TCF/LEF encoding genes. Therefore, we set out to test whether the use of physiological inhibitors of the TCF-β-catenin interaction could drive the expression of

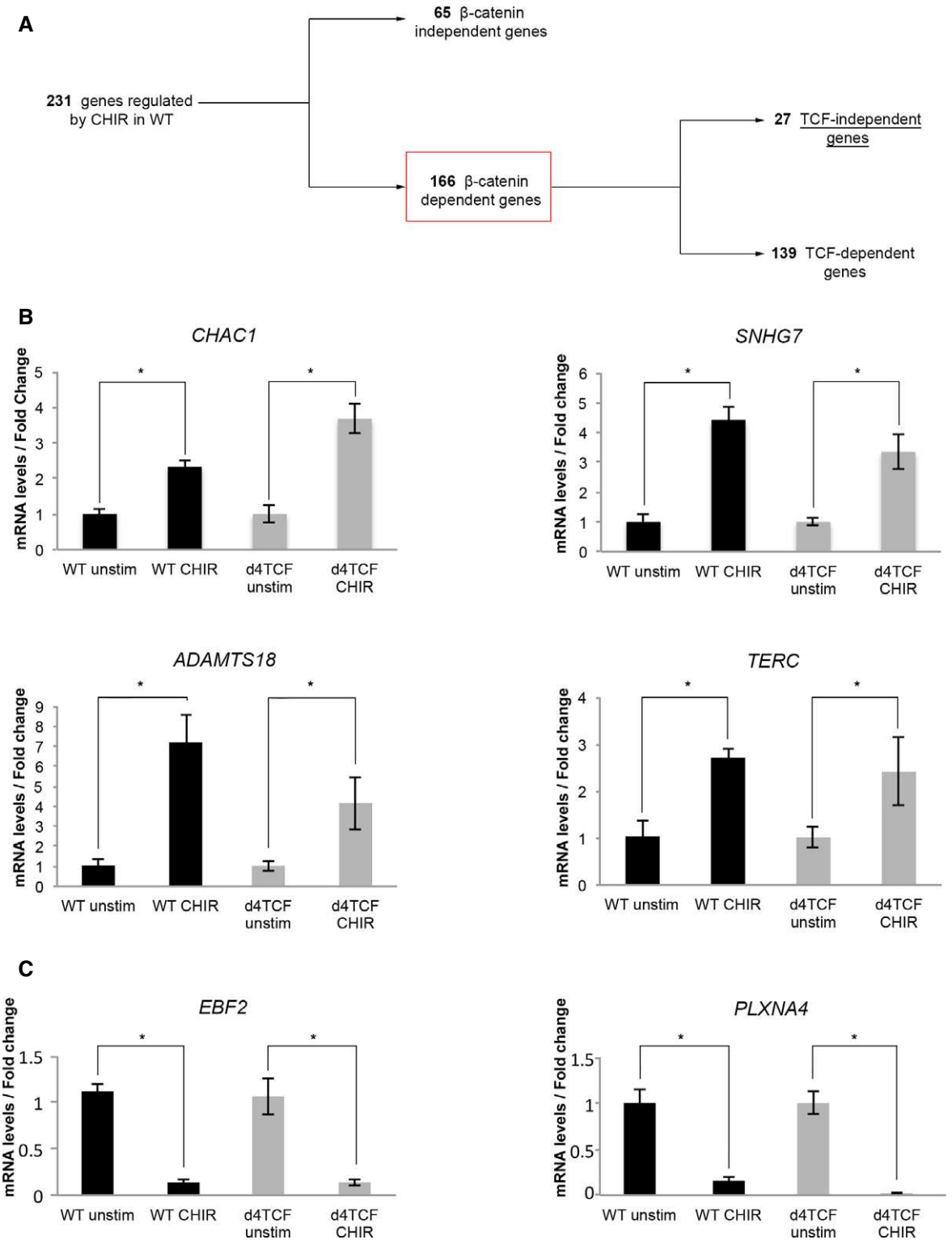


Figure 3. Differential gene expression analysis reveals β-catenin-dependent but TCF/LEF-independent transcriptional changes.

A 231 genes were regulated in wild-type (WT) parental HEK 293T cells upon CHIR treatment (a minimum of twofold change). 166/231 of these transcriptional changes do not occur in dBcat cells and are therefore considered as β-catenin-dependent (red box). 27/166 β-catenin targets are also regulated in d4TCF cells indicating that they are TCF-independent.

B, C RT–qPCR-based validation of selected TCF-independent β-catenin-dependent target genes (B) upregulated or (C) downregulated in d4TCF cells (TCF-independent) treated with 10 μM CHIR or DMSO for 24 h. Data represent the mean ± SEM of averages of three independent experiments ($N = 3$). Samples were compared using Student's *t*-test. Asterisks (*) indicate a $P < 0.05$.

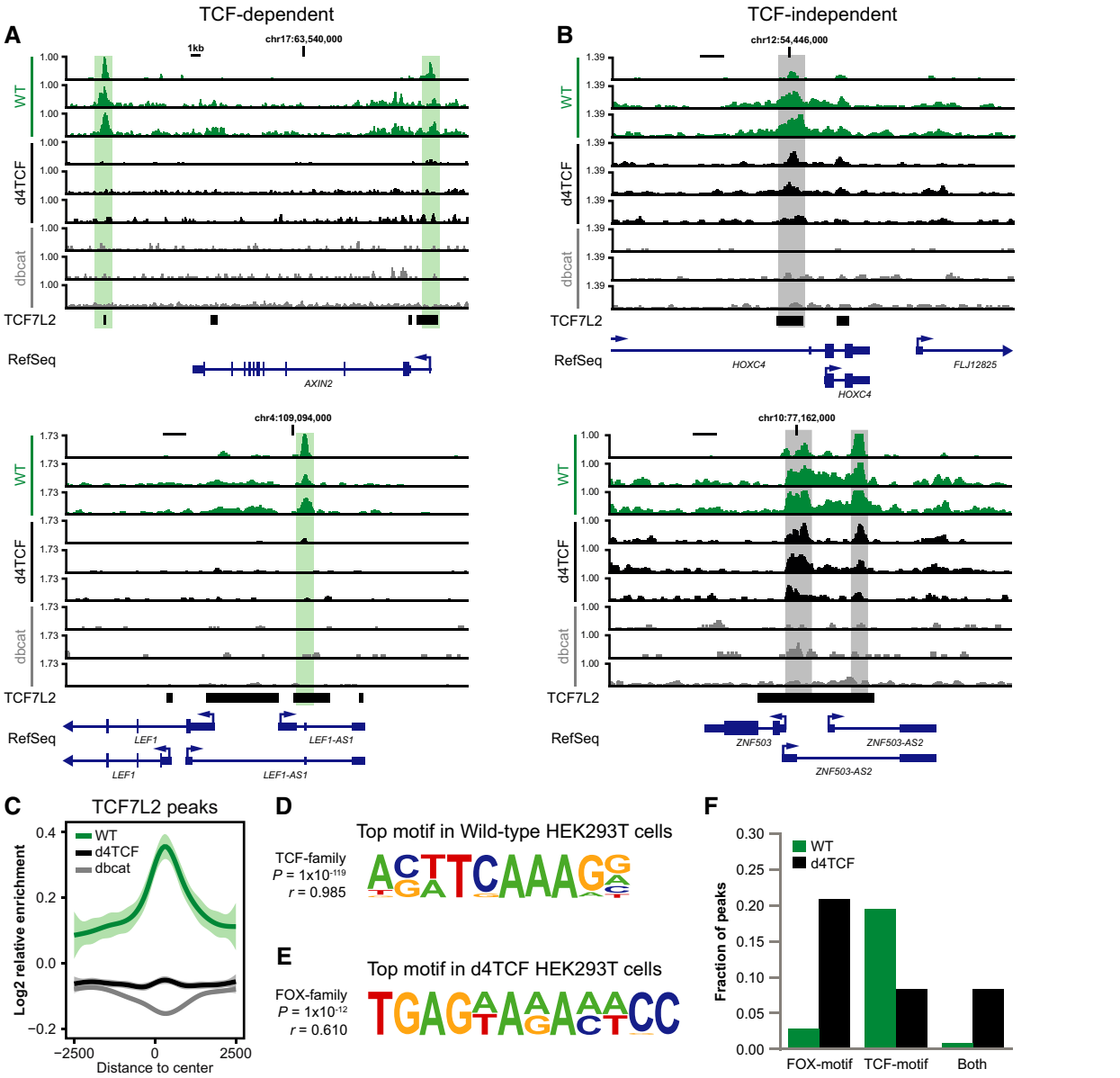


Figure 4. β-Catenin genome-wide occupancy in the presence or in the absence of TCF/LEF transcription factors.

A–C (A, C) Genome browser-based schematic representation of β-catenin peaks in control wild-type (WT), d4TCF or dBcat cells. (A, B) β-Catenin occupancy on regulatory regions of the prototypical Wnt target genes, *AXIN2* and *LEF1* (A), and at the promoter regions of *HOXC4* and *ZNF503* (B). (C) β-Catenin ChIP-seq enrichment over ENCODE TCF7L2 (also known as TCF4) ChIP peaks in HEK 293T cells. The data represent the mean ± 95% C.I. obtained from three independent experiments (N = 3).

D, E Motif analysis of β-catenin peaks in WT (D) and d4TCF cells (E).

F Fraction of peaks containing FOX ("TGAGWARAMWCC"), TCF ("CCTTTGAAST") or both motifs, allowing maximum 1 mismatch.

β-catenin-GHOST targets. We reasoned that blocking the TCF-β-catenin interaction in WT cells would mimic the TCF/LEF quadruple KO condition of d4TCF cells. To achieve this, we overexpressed the inhibitor of β-catenin and TCF (ICAT), a 9-kDa polypeptide that impairs canonical Wnt signalling by competing with TCF/LEF for their interaction to β-catenin (Tago *et al*, 2000). ICAT overexpression inhibited CHIR-mediated *AXIN2* upregulation (Fig EV5A), confirming its ability to block the TCF-β-catenin interaction in HEK 293T cells. Importantly, ICAT overexpression allowed the CHIR-dependent β-catenin-GHOST response: the same set of β-catenin-GHOST targets that could only be upregulated by CHIR in d4TCF cells, was also induced by ICAT overexpression (Fig 5B).

β-Catenin interplay with FOXO transcription factors

We observed a statistical *in silico* enrichment of FOX motifs within the TCF/LEF-independent β-catenin ChIP-seq peaks (Fig 4),

suggesting that at least part of the β-catenin-GHOST response might be co-regulated by FOXO transcription factors. Consistent with this hypothesis, among the top 30 regulated genes within the β-catenin-GHOST signature, we also found known FOXO targets, such as *BCL6*, *BNIP3* and the growth arrest and DNA damage gene *GADD45* (van der Vos & Coffer, 2011). Of note, FOXO transcription factors have been identified as β-catenin cofactors in different cellular contexts (Essers, 2005; Hoogeboom *et al*, 2008). *GADD45*, in particular, has been reported to be a target of FOXO3 and a FOXO4 (van der Vos & Coffer, 2011). We set out to overexpress FOXO3 and FOXO4 in control parental and d4TCF cells, simultaneously trigger β-catenin stabilization with CHIR and monitor the expression of *GADD45*. In WT untreated cells, FOXO4, but not FOXO3, leads to *GADD45* induction (Fig 5C). In d4TCF cells, while FOXO4 alone could mildly induce *GADD45*, the highest induction was obtained when FOXO4 was expressed in CHIR-treated cells (Fig 5D). The FOXO4-dependent induction of *GADD45* was dependent on β-catenin, as it did not occur in dBcat cells (Fig 5D). As previously shown (Essers, 2005; Hoogeboom *et al*, 2008), FOXO4 physically interacts with activated β-catenin, in a dose-dependent manner, also in HEK 293T (Fig 5E). Finally, the addition of FOXO4-specific siRNA —but not control scrambled siRNA—blocked the CHIR-mediated induction of *GADD45* expression (Figs 5F and EV5B and C). Taken together, these results support the notion that, in the absence of TCF/LEF, β-catenin can co-operate with other transcription factors, and FOXO4 plays a role in the transcription of at least a subset of target genes.

Discussion

Here, we generated a series of HEK 293T cell clones lacking all members of the TCF/LEF transcription factor family. This new tool allowed us to probe for the existence of TCF/LEF-independent β-catenin activity (Fig 1). We established a high-confidence list of β-catenin-dependent targets, which comprise exclusively those genes whose expression does not change in the absence of β-catenin when HEK 293T cells are stimulated with CHIR. A significant 15% of this set was regulated by β-catenin irrespectively of the presence or absence of TCF/LEF transcription factors.

Our data convincingly support the paradigmatic view that has been established in the past years of how nuclear Wnt signalling is executed: the vast majority of the β-catenin-dependent transcriptional effects, including the well-established targets such as *AXIN2*, *SP5* and *DKK1*, require the TCF/LEF transcription factors (Figs 2 and 3). Consistently, the genome-wide physical association of β-catenin with DNA is also largely perturbed when TCF/LEF are mutated (Fig 4).

On the other hand, our unbiased approach allowed us to identify a subset of β-catenin targets whose regulation does not require the TCF/LEF proteins. This seems to be in line with previous reports, indicating that β-catenin can bind to alternative non-TCF transcription factors (Essers, 2005; Vadlamudi, 2005; Kaidi *et al*, 2007; Kim *et al*, 2008; Kormish *et al*, 2009). Our study, however, did not allow us to identify all the transcriptional regulators that, in our experimental system, form a complex with β-catenin to regulate TCF/LEF-independent targets. An interesting question for future studies will be to assess whether the TCF-independent β-catenin targets all rely on the activity of a single alternative transcription factor (e.g. FOXO4), or whether different targets are regulated by distinct β-catenin partners. On the other hand, we could identify at least one target gene, *GADD45*, whose expression is driven by the simultaneous action of β-catenin and the Forkhead transcriptional regulator FOXO4 (Fig 5E). This provides the proof-of-principle that alternative mechanisms of action other than the canonical TCF/β-catenin-mediated transduction exist. However, we cannot exclude the possibility that TCF-independent β-catenin targets reflect an indirect effect caused by cytoplasmic functions of β-catenin as part of the destruction complex in the absence of Wnt signals (Nusse & Clevers, 2017). The destruction complex, via the promiscuous activity of GSK3, acts on a plethora of proteins (Taelman *et al*, 2010), and it is possible that the genetic removal of β-catenin may affect the regulation of several genes as a consequence of its impaired activity.

Overall, our observation broadens the spectrum of potential Wnt targets in different tissues: in fact, it paves the way for

Figure 5. β-Catenin drives a GHOST response in the absence of TCF/LEF transcription factors.

A Differential gene expression analysis shows a set of 196 genes being regulated by CHIR in d4TCF cells; several of these changes only appear when TCF/LEF proteins are not present (dark green set). Of the 134 genes that are not regulated by CHIR in dBcat cells (putative β-catenin-dependent genes), 107 require the removal of TCF/LEF factors, in that their regulation only occurs in d4TCF cells. The other 27/134 β-catenin-dependent transcriptional changes match the TCF/LEF-independent target genes previously described in Fig 3A. Their regulation occurs both in the presence and in the absence of TCF/LEF factors. 90/107 of the putative β-catenin-dependent genes, stop being regulated by CHIR in pentaKO cells (β-catenin-GHOST, blue box).

B RT–qPCR validation of the β-catenin-GHOST response. A selection of 7 genes (colour coded) that are activated upon CHIR treatment in d4TCF, or in wild type (WT) in combination with ICAT overexpression, but not in pentaKO cells—indicating that their regulation is dependent on β-catenin but can only occur in the absence of TCF/LEF-β-catenin interaction. The genotype and the treatment for each condition are indicated in the *x*-axis. Error bars show the standard deviation obtained from three independent experiments.

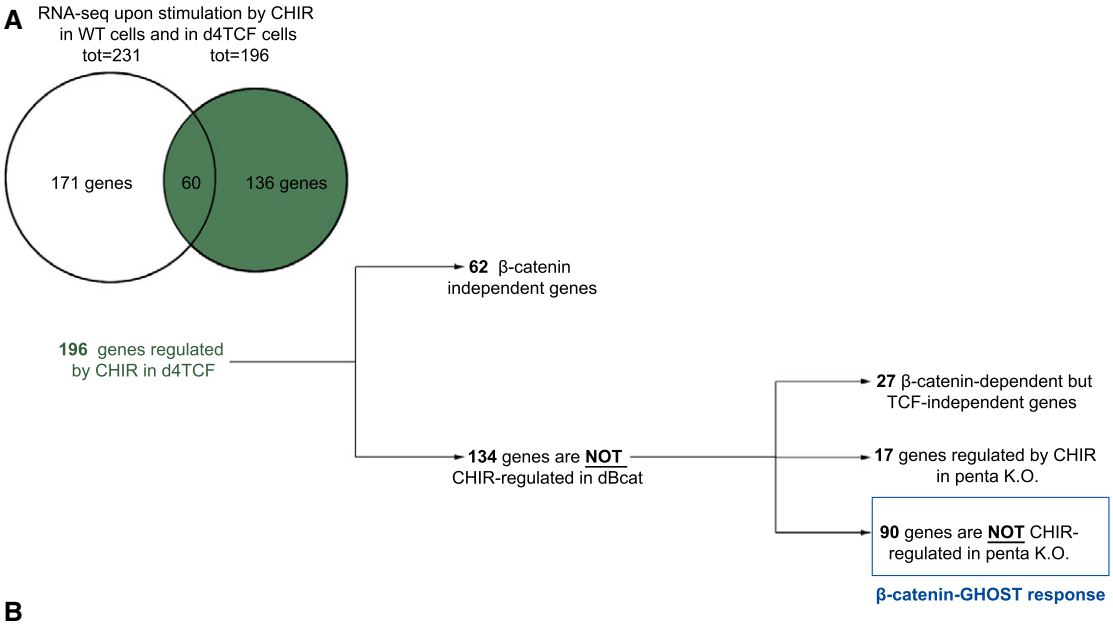
C FOXO4 but not FOXO3 overexpression upregulates the β-catenin-GHOST target *GADD45* both in CHIR-treated WT (black bars) and in d4TCF (grey bars) cells. Samples were compared using Student's *t*-test. Asterisks (*) indicate a $P < 0.05$.

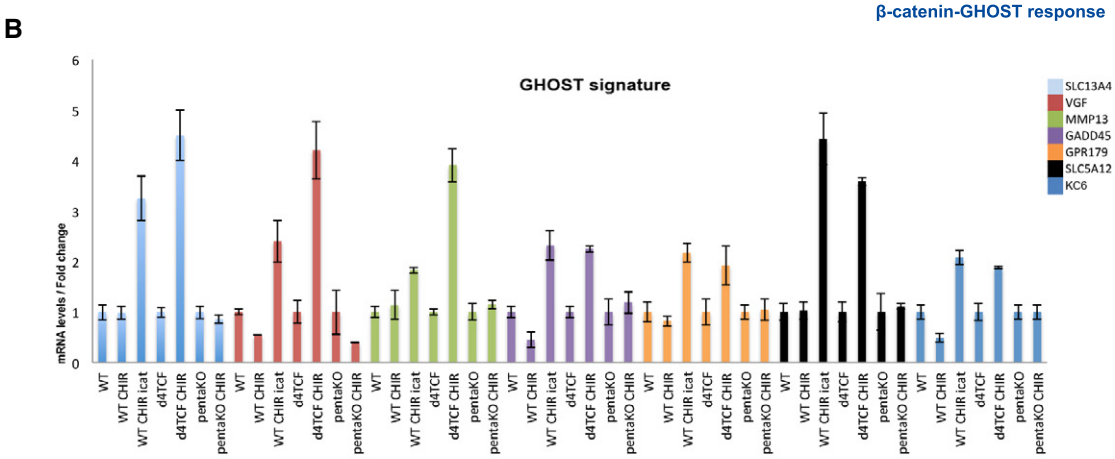
D FOXO4 overexpression induced *GADD45* transcription in WT (black bars) but not in dBcat (grey bars) cells. Samples were compared using Student's *t*-test. Asterisks (*) indicate a $P < 0.05$.

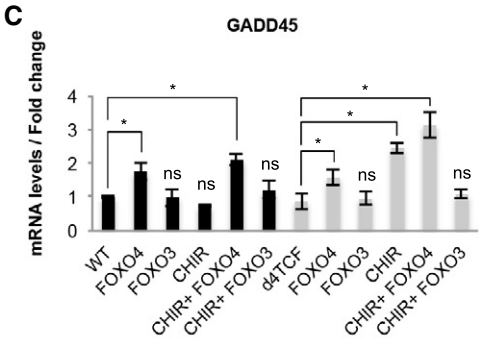
E Endogenous FOXO4, when immunoprecipitated (IP) pulls down β-catenin. Overexpressed FOXO4 increases the amount of β-catenin detected in the immunoprecipitated, indicating physical association.

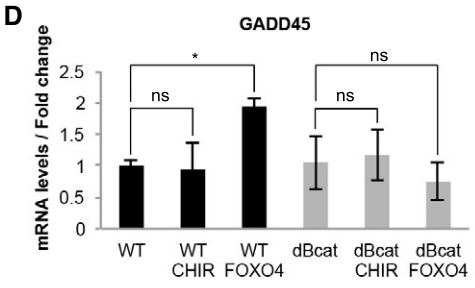
F *GADD45* is upregulated by CHIR in d4TCF cell; this positive transcriptional regulation is blocked by FOXO4-specific siRNA but not control scrambled siRNA. All experiments were done at least three times ($N = 3$). Samples were compared using Student's *t*-test. Asterisks (**) indicate a $P < 0.02$; ns, non-statistically significant change was observed.

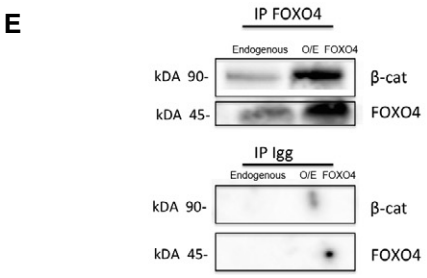
Source data are available online for this figure.











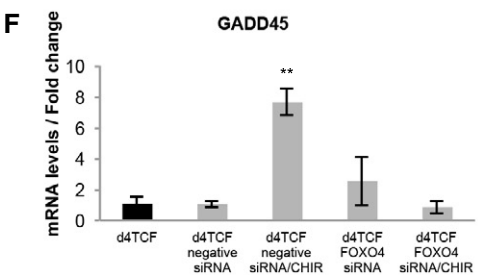


Figure 5.

understanding how β-catenin stabilization could regulate different sets of targets within the same cells or in different tissues, namely by complexing with different partners capable of recognizing a wide range of DNA motifs present in gene regulatory regions.

Interestingly, among the TCF-independent β-catenin target loci (i.e. ChIP-seq peaks), we could identify peaks whose size (i.e. the fold enrichment) was unchanged upon loss of TCF/LEF (Fig 4C): we interpret this as an entirely TCF-independent binding event. On the other hand, other peaks were decreased in size, but still present, such as at the *ZNF503* locus (Fig 4C). It is possible that this second binding behaviour reflects the fact that β-catenin relies on the presence of TCF/LEF in co-operation with other transcription factors, such as FOXO4. This view is supported by the observation that *ZNF503* and its antisense transcript require TCF/LEF for their transcriptional regulation (Table EV1). Importantly, also, all β-catenin peaks in d4TCF cells containing a TCF motif also contained a FOX binding motif (Fig 4F). In this scenario, the removal of TCF/LEF could attenuate, but not fully abolish, β-catenin occupancy at this region, and transcriptional regulation of the affected gene. Collectively, these data constitute powerful evidence that the association of β-catenin to a subset of genomic regions cannot be entirely explained by its association with TCF/LEF proteins and must require additional coupling with alternative DNA-binding partners.

Possibly the most interesting discovery we made was the observation that in a cellular context devoid of TCF/LEF proteins, β-catenin acquires an entirely new set of transcriptional targets (Fig 5). We provide evidence for this at the RNA level, by showing that a new group of genes is transcriptionally regulated by β-catenin, upon CHIR treatment, only when TCF/LEF genes are mutated. These β-catenin targets are not only TCF-independent, but they require the inhibition of the TCF-β-catenin interaction, or the loss of TCF/LEF expression. It is possible that, in normal conditions, the β-catenin nuclear protein is present in limiting amount, and DNA-β-catenin interaction is predominantly mediated by TCF/LEF, preventing the co-operation with other potential protein partners. The removal of TCF/LEF, however, might release β-catenin to bind to different interactors. Of course, we are aware that the loss of function of all *TCF/LEF* encoding genes might lead to an artefactual scenario that does not necessarily parallel a physiologically relevant condition. Additional studies are required to address whether the β-catenin-GHOST response can take place during organismic development, homeostasis or, perhaps in disease condition, upon physiological Wnt stimulation. Interestingly enough, the expression of ICAT, a small protein physiologically capable of impairing the β-catenin–TCF interaction *in vivo*, can mimic the genetic removal of TCF/LEF genes (Fig 5B). It appears therefore that, when β-catenin is "liberated" by the affinity with TCF/LEF proteins, it becomes free to regulate new sets of targets by forming new partnerships. In this light, we speculate that the many physiologically occurring peptides, like ICAT itself (Hossain *et al*, 2008), or the dominant negative isoforms of LEF-1 and TCF7 (Yokoyama *et al*, 2010; Sprowl-Tanio *et al*, 2016), might function *in vivo* as molecular switches capable of unleashing β-catenin from its canonical role, and permitting the activation of the β-catenin-GHOST response. It is also plausible that the β-catenin-GHOST response is a variable entity that strictly depends on the tissue context: different sets of transcription factors might be present in different cell types. β-Catenin has a recognized role as a scaffold protein (Valenta *et al*, 2012): in canonical Wnt signalling, β-catenin can tether a cohort of transcriptional regulators to WREs and functionally couple them with the Pol II complex. Perhaps β-catenin can act as a promiscuous transcriptional effector, capable of connecting the function of other transcription factors with the general transcriptional machinery.

Finally, our work brings forward the tantalizing hypothesis that other developmental signalling pathways might possess a hidden response when the key transcription factors are limiting, not expressed or prevented from interacting with their upstream pathway component (e.g. GLI proteins in SHH signalling; Rimkus *et al*, 2016). Uncovering potential further GHOST responses might lead to a new understanding of how downstream effectors of signalling cascades modulate different tissue-specific responses.

Materials and Methods

Cell culture

Human embryonic kidney (HEK) 293T cells were cultured in media consisting of DMEM, high glucose (41966-029 Gibco) supplemented with 10% foetal bovine serum (Gibco), 1× penicillin–streptomycin according to the manufacturer's recommendation.

CRISPR guide RNAs

Guide RNAs (sgRNAs) were designed with the online tool: Optimized CRISPR design (http://crispr.mit.edu/) and were cloned into the pSpCas9(BB)-2A-Puro (PX459) (Addgene plasmid ID: 48139) as previously described in Ran *et al* (2013). All sgRNAs used are listed in Table EV4.

Generation of K.O. cell lines using CRISPR/Cas9

Two single-guide RNAs, targeting simultaneously either two exons or an exon and an intron of each TCF/LEF (see table), respectively, were cloned separately into pSpCas9(BB)-2A-Puro (PX459) in order to sequentially ablate all full-length TCF/LEF isoforms. The constructs were co-transfected transiently in HEK 293T cells using Fugene according to the manufacturer's recommendation, and 24 h after transfection, puromycin selection was applied for an additional 48 h. Cells were then split and seeded at clonal density. Clones from single cells were manually picked and analysed by Western blot for the expression of TCFs. The same approach was used also for the generation of the beta-catenin K.O. Table shows the gRNA sequence and the corresponding target region.

Western blot analyses

Cell lysates were mixed with LDS sample buffer supplemented with sample reducing agent (invitrogen) and boiled for 5 min at 95°C. 10–15 mg of protein/lane was run through 10% Bis–Tris gels (120 V, 90 min). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes with a Bio-Rad Mini Trans-Blot System (35 V, 3 h). Membranes were blocked with 5% milk/PBS-T (phosphate-buffered saline supplemented with tween 20) for 45 min in room temperature (RT) and incubated with primary antibodies diluted in 5% bovine serum albumin/PBS-T overnight at 4°C. Blots were

washed with PBS-T (four times, 15 min/wash) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1 h, RT). After four washes with 15 min in PBS-T, blots were incubated in WesternBright Quantum (Advansta) and imaged by using a Fusion SL imaging System (Vilmer). The following primary antibodies were used for Western blot: mouse anti-a-tubulin (T6074, Sigma-Aldrich), rabbit anti-TCF7 (C63D9, Cell Signaling), rabbit anti-LEF1 (C12A5, Cell Signaling), rabbit anti-TCF7L1 (D15G11, Cell Signaling), rabbit anti-TCF7L2 (C48H11, Cell Signaling), rabbit anti-β-catenin (SantaCruz sc-7199). Horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies for Western blotting were purchased from Jackson.

Plasmid list

The following plasmids were obtained from Addgene: pSpCas9(BB)-2A-Puro (PX459), pcDNA3flagFKHRL1 (FOXO3) (#10709), pcDNA-HA-TCF1 (TCF7) (#40620), pcDNA-myc-TCF4 (TCF7L2) (#16512), pc-DNA3hE47 (TCF7L1) (#16059). The pCMV-ICAT was a kind gift from Prof. Christian Mosimann. The FOXO4 expressing plasmid was kindly provided by Prof. P.J. Coffer. The LEF1 expressing plasmid was kindly provided by Prof. Marc de la Roche.

qRT–PCR

Total RNA (1 μg) isolated using the PureLink RNA Mini Kit (Thermo Fisher Scientific) was used to generate cDNA with Transcription High Fidelity cDNA Synthesis Kit (Roche) and then treated with DNA-free Kit (Thermo Fisher Scientific). SYBR Green SuperMix (Bio-Rad) was used for qPCRs using 3 μl of diluted cDNA (1 μg RNA equivalent in 100 μl). GAPDH was used as the reference gene. QuantStudio 3 (Thermo Fisher Scientific) instrument and software were used to determine relative gene expression levels using the delta–delta Ct method. Primer sequences were designed using Roche's online primer design software (https://lifescience.roche.com/en_ch/brands/universal-probe-library.html) or were obtained from prior publications and are listed in Table EV5. Statistical analyses (two-tailed Student's *t*-test) was performed in Microsoft Excel considering both independent experiments and technical repetitions. Each experiment was performed at least three times.

TCF/LEF reporter assays

HEK 293T cells were co-transfected with the constructs 8XTOPFlash or 8XFOPFlash, driving firefly luciferase production, and pRL-CMV, driving constitutive expression of Renilla luciferase for normalization (Promega). For experiments performed with 10 μM CHIR99021, 293T cells were resuspended directly into the appropriate media. Cells were washed twice with PBS 48 h following transfection and lysed with 1× passive lysis buffer (Promega). The firefly and luciferase activities were measured using a 96-well-based luminometer as per the manufacturer's instructions (Promega Dual-Light System).

siRNA transfection

For the FOXO4 siRNA transfections the *Dharmacon RNAi, Gene expression & Gene Editing* reagents were used. HEK 293T cells were plated in 24-well plate the day before transfection (60% confluency) in antibiotic-free complete medium. Cells were incubated overnight at 37°C with 5% CO_2. Negative control (ON-TARGET plus non-targeting pool D-001810-10-05) and test siRNAs (ON-TARGET plus SMART pool FOXO4 siRNA L-003016-00-0005) were used in 5 nM final concentration. The transfections were performed according to the *DharmaFECT1* transfection reagent protocol. Transfection medium was replaced with complete new medium, supplemented with 10 μM final concentration CHIR, after 24 h to reduce cytotoxicity and to stimulate the Wnt pathway. 24 h later, cells were harvested and analysed with qRT–PCR.

Chromatin immunoprecipitation

Cells were treated with 10 μM CHIR or 10 μM DMSO for 24 h prior to cross-link. Ca. 50×10^6 HEK cells per samples were cross-linked in 20 ml PBS for 40 min with the addition of 1.5 mM ethylene glycol-bis(succinimidyl succinate) (Thermo Scientific, Waltham, MA, USA), for protein–protein cross-linking (Schuijers *et al*, 2014), and 1% formaldehyde for the last 20 min of incubation, to preserve DNA–protein interactions. The reaction was blocked with glycine and the cells were subsequently lysed in 1 ml HEPES buffer (0.3% SDS, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES). Chromatin was sheared using Covaris S2 (Covaris, Woburn, MA, USA) for 8 min with the following set up: duty cycle: max, intensity: max, cycles/burst: max, mode: Power Tracking. The sonicated chromatin was diluted to 0.15% SDS and incubated overnight at 4°C with 10 μg of anti-β-catenin (SantaCruz sc-7199; Cell Signaling) and 50 μl of protein A/G magnetic beads (Upstate). The beads were washed at 4°C with wash buffer 1 (0.1% SDS, 0.1% deoxycholate, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES), wash buffer 2 (0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES), wash buffer 3 (0.25 M LiCl, 0.5% sodium deoxycholate, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES) and finally twice with Tris EDTA buffer. The chromatin was eluted with 1% SDS, 0.1 M $NaHCO_3$, de-cross-linked by incubation at 65°C for 5 h with 200 mM NaCl, extracted with phenol–chloroform and ethanol-precipitated. The immunoprecipitated DNA was used as input material for DNA deep sequencing.

ChIP-seq data analysis: ChIP-seq reads were mapped using the Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) onto the UCSC hg19 reference human genome. Peaks were called against Input controls using MACS2 (https://github.com/taoliu/MACS/) (Zhang *et al*, 2008) (using flag –nomodel) and peaks overlapping known problematic ChIP regions from ENCODE (wgEncodeHg19ConsensusSignalArtifactRegions.bed) were excluded. Next, peaks identified in the majority of replicates (within 500 bp from peak centre) were kept (Yang *et al*, 2014). Motif enrichment analysis of peaks was performed using Homer2 (PMID: 20513432). DeepTools (Ramírez *et al*, 2016) was used to calculate genome-wide coverage against Input controls (bamCompare –e 200 –ignoreDuplicates) and plot enrichment over features (plotHeatmap). Public ENCODE ChIP peaks used as features were obtained for ENCFF002CRP (TCF7L2, also known as TCF4), and peaks within 500 bp for each file were merged. For merged peaks, randomly sampled regions were obtained and used to normalize for overall

signal levels between experiments. The data have been deposited at the ArrayExpress database, accession number E-MTAB-7028.

Transcriptome analyses

RNA from HEK 293T cells was extracted using TRIzol reagent (Ambion); libraries were prepared with TruSeq RNA stranded library preparation and sequenced on NextSeq 500, 1×75 bp. Reads in FASTQ format from the 18 samples (six conditions with three biological replicates each) were quantified at the gene level with featureCounts (Liao *et al*, 2014) using the built-in human EntrezGene annotation. The count table was delivered to edgeR (Robinson *et al*, 2010) for the differential expression analysis using the GLM functionality (McCarthy *et al*, 2012) (R version 3.3.0, edgeR version 3.14.0). To determine differential expression, pairwise contrasts of interest were designed, a likelihood ratio test was calculated, and a Benjamini–Hochberg multiple testing correction (estimated FDR of 5%) with minimum absolute fold change of 1.5 was applied. Heat maps were generated using the pheatmap package. The data have been deposited at the ArrayExpress database, accession number E-MTAB-7029.

Expanded View for this article is available online.

Acknowledgements
We are immensely grateful to George Hausmann for comments on the project execution and manuscript writing, Dario Zimmerli and Tomas Valenta for invaluable scientific advice, P.J. Coffer for providing the FOXO expressing plasmids and Eliane Escher for technical support. N.D. was supported by an EMBO long-term fellowship and C.C and K.B. by fundings from the University of Zurich. The Knut and Alice Wallenberg Foundation is acknowledged for generous support to C.C.

Author contributions
ND performed the experiments, analysed the data and prepared the figures. MDR and FL analysed the RNA-seq data. AL and CEN performed ChIP-seq bioinformatics analyses and assisted in figure preparation. CC performed the ChIP experiments and analyses, designed and interpreted the experiments, and wrote the manuscript. KB supervised and assisted the research team, provided the initial idea and interpreted the data. All authors critically revised the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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